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Hierarchical order of distinct autoantibody spreading and progression to type 1 diabetes in the TEDDY Study

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Abstract

Objective: The first-appearing β-cell autoantibody has been shown to influence risk of type 1 diabetes. Here, we assessed risk of autoantibody spreading to the second-appearing autoantibody and further progression to clinical disease in the Environmental Determinants of Diabetes in the Young study.

Research Design and Methods: Eligible children with increased HLA-DR-DQ genetic risk for type 1 diabetes were followed quarterly from age 3 months up to 15 years for development of a single first-appearing autoantibody (GADA, IAA or IA-2A) and subsequent development of a single second-appearing autoantibody and progression to type 1 diabetes. Autoantibody positivity was defined as positivity for a specific autoantibody at two consecutive visits confirmed in two laboratories. ZnT8A was measured in children who developed another autoantibody.

Results: There were 608 children who developed a single first-appearing autoantibody (IAA, n=282 or GADA, n=326) with a median follow-up of 12.5 years from birth. The risk of a second-appearing autoantibody was independent of GADA versus IAA as a first-appearing autoantibody (adjusted-HR=1.12, 95%CI=0.88-1.42, *P*=0.36). Second-appearing GADA, IAA, IA-2A or ZnT8A conferred an increased risk of type 1 diabetes compared to children who remained single autoantibody positive (IAA- or GADA-second: adjusted-HR=6.44 95%CI=3.78-10.98; IA-2A-second: adjusted-HR=16.33 95%CI=9.10-29.29, *P*<0.0001; ZnT8A-second: adjusted-HR=5.35 95%CI=2.61-10.95, *P*<0.0001). In children who developed a distinct second autoantibody, IA-2A (adjusted-HR=3.08 95%CI=2.04-4.65, *P*<0.0001) conferred a greater risk of progression to type 1 diabetes as compared to GADA or IAA. Additionally, both a younger

initial age at seroconversion and shorter time to the development of the second-appearing autoantibody increased the risk for type 1 diabetes.

Conclusions: The hierarchical order of distinct autoantibody spreading was independent of the first-appearing autoantibody type, age-dependent and augmented the risk of progression to type 1 diabetes.

β-cell autoantibodies are predictors of type 1 diabetes (1; 2) and, currently, the most reliable indicator of pre-clinical disease. The presence of two or more autoantibodies against the four major autoantigens (insulin, glutamate decarboxylase, insulinoma-associated protein 2 and zinc transporter-8) have been shown to confer the highest risk of type 1 diabetes and was recently defined as Stage 1 type 1 diabetes (3). β-cell autoantibodies develop prior to and usually persist over time in the progression to clinical onset of type 1 diabetes (Stage 3). The age at appearance of specific β-cell autoantibodies varies (4-6). Autoantibodies to glutamate decarboxylase (GADA) or insulin (IAA) predominate as first-appearing autoantibodies (7; 8). IAA appearance peaks within the first 2 years of life. In contrast, GADA appears between ages 3-5 years in children with either increased genetic risk or family history of type 1 diabetes (7-9). Insulinoma-associated protein 2 (IA-2A) and zinc transporter-8 (ZnT8A) autoantibodies generally appear after the initial islet autoantibody seroconversion (6; 8; 10; 11) in more advanced stages of the disease process (3; 12-14).

Recent studies have focused on the appearance of the first-appearing autoantibody (7; 8) with limited discussion of the specific second-appearing autoantibody (8). The Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study (8) reported that IAA as a second-appearing autoantibody peaked in the first five years of life and followed a similar pattern to first-appearing GADA; whereas, GADA peaked within the first two years of life and mostly occurred after IAA. More recently, the DIPP study reported that the initial age at seroconversion was only associated with IAA-initiated seroconversion and further progression to type 1 diabetes (15). However, due to a varied autoantibody screening interval, further exploration after the first two years of life was limited. Other studies assessing the presence of autoimmunity and combinations of autoantibodies were carried out where the actual order of appearance could not be distinguished

due to annual screening (16; 17) or capture was at the time of or just prior to diagnosis (1; 11; 18-22). These previous studies assessing risk from single to multiple autoantibody showed increased risk was associated with a younger age, HLA-DR-DQ and IAA as first-appearing autoantibody (16; 17). In addition to HLA class II risk, a recent study from the Belgian Diabetes Registry (23) reported that specific HLA class I alleles accelerated progression from multiple autoantibody positivity to type 1 diabetes in those <40 years with a family history of type 1 diabetes. Specifically, HLA-A*24 was associated with an accelerated progression in HLA-DQ8^{+} relatives who developed either IA-2A or ZnT8A; whereas, HLA-B*18 was associated with accelerated progression in HLA-DQ2^{+} relatives who developed both IAA and GADA (23). Moreover, non-HLA single nucleotide polymorphisms (SNP), such as those in the *INS-VNTR* gene region (rs1004446) and protein tyrosine phosphatase non-receptor type 22 (*PTPN22*, rs2476601) have been shown to associate with a decreased and increased risk of progression to islet autoimmunity and type 1 diabetes, respectively (24; 25). Thus, understanding the order and time of appearance of each distinct autoantibody will allow for better stratification of risk.

The aims of this study were 1) to assess the risk of developing a distinct second islet autoantibody in children at increased genetic risk for type 1 diabetes who newly seroconverted for either IAA-only or GADA-only as first-appearing autoantibodies; and, 2) to determine the risk associated with the specific target of the first- and second-appearing autoantibody combination on further progression to clinical type 1 diabetes.

Research Design and Methods

Study population. The Environmental Determinants of Diabetes in the Young (TEDDY) is a prospective cohort study of children at increased genetic risk for type 1 diabetes funded by the National Institutes of Health. The TEDDY study seeks to identify environmental causes of type 1

diabetes. There are six clinical research centers - three in the US: Colorado, Georgia/Florida, Washington and three in Europe: Finland, Germany, and Sweden. The high-risk genotypes for subjects screened from the general population with no family history of type 1 diabetes (89%) were as follows: DRB1*04-DQA1*03-DQB1*03:02/DRB1*03-DQA1*05-DQB1*02:01 (DR3/4-Q2/8), DRB1*04-DQA1*03-DQB1*03:02/DRB1*04-DQA1*03-DQB1*03:02 (DR4/4-DQ8/8), DRB1*04-DQA1*03-DQB1*03:02/DRB1*08-DQA1*04-DQB1*04:02 (DR4/8-DQ8/4) and DRB1*03-DQA1*05-DQB1*02:01/DRB1*03-DQA1*05-DQB1*02:01 (DR3/3-DQ2/2) and six additional genotypes in first degree relatives (FDR) of those with a family history of type 1 diabetes as previously described (26). There were 8,676 children enrolled from September 2004 to February 2010 and currently followed prospectively from age 3 months to 15 years with study visits every 3 months until 4 years and every 3 or 6 months, thereafter, depending on autoantibody positivity. All children who are persistent positive for any autoantibody are followed every 3 months until the age of 15 years or onset of type 1 diabetes. Detailed study design and methods have been published previously (26-28). The protocol was approved by Institutional Review Boards at participating centers and all participants provided written informed consent before participation in the genetic screening and enrollment.

β-cell Autoantibodies. β-cell autoantibodies to IAA, GADA or IA-2A were measured in two laboratories by radiobinding assays (27; 29). In the U.S., all sera were assayed at the Barbara Davis Center for Childhood Diabetes at the University of Colorado Denver; in Europe, all sera were assayed at the University of Bristol, in the U.K. Both laboratories reported high sensitivity, specificity, and concordance (29; 30). All positive β-cell autoantibodies and 5% of negative samples were re-tested in the other reference laboratory and deemed confirmed if concordant. Autoantibody positivity (persistent confirmed) was defined as specific autoantibody presence on

≥2 consecutive visits 3 months apart and confirmed in two TEDDY laboratories. The date of positivity was the draw date of the first sample of the two consecutive positive samples which deemed a child persistent confirmed positive for a specific autoantibody. Age of seroconversion was the age of the child on the initial date of seroconversion to the first persistent confirmed autoantibody. Age of second autoantibody seroconversion was the age of the child on the date of autoantibody seroconversion of a second persistent confirmed autoantibody. Stage 1 type 1 diabetes was defined as presence of multiple autoantibodies (3). Symptomatic type 1 diabetes (Stage 3) was defined according to American Diabetes Association criteria for diagnosis (3; 31). There were 8,502 of the 8,676 children eligible for this analysis; 174 were ineligible for this analysis and excluded due to not having an autoantibody test result (n=54) or were deemed HLA-DR-DQ ineligible based on study criteria (n=120) (**Supplemental Figure S1, Supplemental Table S1**). As the focus of this analysis was to examine autoantibody spreading, the analyses were restricted to the development of the first-appearing IAA-single (n=282) or GADA-single (n=326) as of 31 December 2018 and followed through 31 December 2019; excluding children who developed multiple autoantibodies (≥ 2 autoantibodies) at their initial seroconversion (n=156) or IA-2A-only (n=15), or ZnT8A-only (n=12). There were 608 children included in these analyses.

Zinc transporter 8 autoantibodies. ZnT8A radioimmunoassay was performed as previously described (19). The ZnT8 protein was produced by in vitro transcription and translation (Promega TNT kit, Madison, WI) and labeled with [35S]methionine (Perkin Elmer, Waltham, MA). [35S]ZnT8 (20,000 cpm) was mixed and incubated overnight at 4 °C with 2 µl of serum in an assay buffer (20 mM Tris HCl, pH 7.4, supplemented with 150 mM NaCl, 0.1% (w/v) BSA, 0.15% (v/v) Tween-20 and 0.1% (w/v) sodium azide) at a final 1:25 dilution. Autoantibody-

bound antigen was precipitated with 25 µl of 50% protein A-Sepharose (GE HealthCare, Piscataway, NJ) in an opaque 96-well filtration plate (Corning, Tewksbury, MA) and washed with two cycles of four washes per each cycle of cold assay buffer using a vacuum-operated 96-well plate washer (BioTek, Winooski, VT). After washing, scintillation fluid, MicroScint-20 (Perkin Elmer, Waltham, MA), was added directly to the 96-well plate, and radioactivity was counted on a TopCount 96-well plate β-counter (Perkin Elmer, Waltham, MA). The results are expressed as an index (index = (sample CPM – negative control CPM)/(positive control CPM – negative control CPM). The upper limit of normal (0.020) was established as the 99th percentile from receiver operating characteristic curves in 100 healthy control subjects and 50 patients with new onset diabetes. In the most recent Islet Autoantibody Standardization Program (2017) workshop, the sensitivity and specificity were 66 and 100%, respectively. ZnT8A was run on all available samples at the TEDDY Barbara Davis Laboratory, if a child was deemed antibody positive for IAA, GADA and/or IA-2A (any number of autoantibodies, any autoantibody, persistence or confirmation not required) at a visit. Persistent ZnT8A was defined as the presence of ZnT8A on ≥2 consecutive visits 3 months apart in a single laboratory. Protocol compliance for measurement of ZnT8A prior to the first autoantibody positive sample in children with persistent IAA, IA-2A or GADA was 99%. For children who did not meet protocol compliance (n=9), the median months following the first autoantibody positive sample was 3.7 months.

Statistical Analysis. Cumulative incidence of the appearance of the second autoantibody and the risk of progression to type 1 diabetes were examined in Kaplan-Meier analyses. Multivariable proportional hazards (PH) models were applied to assess both the cause-specific risk of autoantibody spreading to a second-appearing autoantibody and the risk of progression to type 1 diabetes from the second-appearing autoantibody. Each specific second-appearing autoantibody

was also examined as a time-varying predictor on risk of type 1 diabetes. To ensure that the second-appearing autoantibody was compared to single autoantibody positive children in each risk set, the other single second-appearing autoantibodies or multiple second-appearing autoantibodies (two autoantibodies appearing during the same 3-month interval) were censored at the time of their appearance as defined above. The strength of the associations with type 1 diabetes was described by hazard ratios (HRs) and with second-appearing autoantibodies by cause-specific hazard ratios (CHRs) with 95% confidence intervals (CIs). All multivariable analyses were adjusted for HLA-DR-DQ, gender, family history of type 1 diabetes, age at initial seroconversion, and first-appearing autoantibody (IAA or GADA). The following also considered for adjustment (and remained in the final model if $P < 0.10$) were HLA Class I alleles (HLA-A*24:X, -B*18:X:X) (23), *SLC30A8* (rs13266634) (32), SNPs previously shown to be associated with islet autoimmunity and type 1 diabetes in TEDDY (*INS* (rs1004446), *PTPN22* (rs2476601), *ERBB3* (rs2292239), *SH2B3* (rs3184504), *TNFAIP3* (rs2327832), rs1534422, rs10517086) (5; 24; 25; 33), and characteristics found to be associated with islet autoimmunity and type 1 diabetes (34-37), unless otherwise stated. Country of residence was included in all models as a stratified variable. Adjustments for population stratification were made by using the top two principal components from the Immunochip SNP data as covariates in the PH model (38). Data were analyzed using the Statistical Analysis System Software (Version 9.4, SAS Institute, Cary, NC) and GraphPad PRISM 7.04 (GraphPad Software Inc., San Diego, CA) for figures. Two-tailed *P* values less than 0.05 were considered significant.

Results

As of 31 December 2018, 608 of the 8,502 enrolled children with HLA-DR-DQ genetic risk for type 1 diabetes in TEDDY developed a single positive IAA or GADA at their initial

seroconversion and met the inclusion criteria for this analysis: IAA-only (n=282), GADA-only (n=326). Of those who developed first-appearing IAA- or GADA-only (n=608), 272 (44.7%) remained single autoantibody positive for the duration of follow-up through 31 December 2019 and 10.3% (n=28/272) of these single positive children progressed to type 1 diabetes within 0.95 (0.28-3.37) years; 55.3% (n=336/608) developed another autoantibody(s) and 53.0% (n=178/336) of these children progressed to type 1 diabetes within 3.53 (1.56-5.83) years. The median (interquartile range (IQR)) age at initial GADA- or IAA-only seroconversion was 4.28 (2.27-7.51) and 1.83 (1.00-3.82) years, respectively. The median (IQR) follow-up from the initial seroconversion was 5.7 (3.2-8.7) years with the majority (98%) followed for $\geq$2 years.

Time from first- to second-appearing autoantibody

The characteristics of the children by specific second-appearing autoantibody are shown in **Supplemental Table S2** and the age descriptive patterns of the second-appearing autoantibody are shown in **Supplemental Figure S2**. GADA as a second-appearing autoantibody developed at a median (IQR) of 3.7 (3.0-9.7) months after the first-appearing autoantibody, IAA as second-appearing autoantibody developed at a median of 5.9 (3.0-25.9) months, IA-2A as a second-appearing autoantibody developed at a median of 6.9 (3.7-17.6) months, and ZnT8A as a second-appearing autoantibody developed at a median of 13.3 (6.3-23.7) months after the first-appearing autoantibody. The overall median (Q1-Q3) months after the first-appearing autoantibody to the appearance of the second autoantibody was 6.8 (3.2-17.0). There was no significant difference in the time from the first- to second-appearing autoantibody by the type of first-appearing IAA or GADA, taking into account the age at the initial seroconversion.

Risk factors associated with development of a second-appearing autoantibody

Known type 1 diabetes risk factors were examined for univariate association with developing a second autoantibody, **Supplemental Table S3**. Family history of type 1 diabetes was positively associated with the development of a second autoantibody; while, having a minor allele in *INS-VNTR* gene region (rs1004446) or HLA-DR3/3 was inversely associated with developing IAA or GADA as a second autoantibody. Age at initial seroconversion was inversely associated with the development of a second autoantibody in multivariable analyses, **Table 1**. Of interest, the risk of developing a second autoantibody declined with increasing age at initial seroconversion (for each additional month older, adjusted-HR=0.986, 95%CI=0.983-0.990, *P* <0.0001).

From the time of the first-appearing autoantibody, the risk of appearance of any second autoantibody did not differ if GADA or IAA was the first-appearing autoantibody (adjusted-HR=1.12, 95%CI=0.88-1.42, *P*=0.36), independent of age at initial seroconversion. Further assessment did not find any specific second autoantibody associated with a specific first-appearing autoantibody (IAA or GADA) after the Bonferroni correction for significance at *P*<0.008 for multiple comparisons, **Figure 1, Panels A and B**. Overall, there was no significant difference based on either GADA-only or IAA-only as the first-appearing autoantibody on the risk of developing IA-2A (adjusted-CHR=1.23, 95%CI=0.69-2.20, *P*=0.48) or ZnT8A (adjusted-CHR=1.62, 95%CI=0.91-2.86, *P*=0.09) as a second-appearing autoantibody.

Influence of a second autoantibody on risk of progression to type 1 diabetes

We next asked if the appearance of the second autoantibody (time-varying predictor) from the time of the first-appearing autoantibody influenced progression to type 1 diabetes. Children who developed a distinct second-appearing autoantibody had an increased risk of progression to type 1 diabetes as compared to children who remained single autoantibody

positive (IAA- or GADA-second: adjusted-HR=6.44 95%CI=3.78-10.98, *P*<0.0001; IA-2A-second: adjusted-HR=16.33 95%CI=9.10-29.29, *P*<0.0001; ZnT8A-second: adjusted-HR=5.35 95%CI=2.61-10.95, *P*<0.0001) from age of first-appearing autoantibody, adjusting for age at initial seroconversion, sex, family history of type 1 diabetes, HLA-DR-DQ, *INS-VNTR* gene region (rs1004446) and type of first-appearing autoantibody. No other HLA Class I alleles or non-HLA SNPs assessed were found to significantly associate with developing a specific second autoantibody.

Risk factors associated with type 1 diabetes in children with multiple autoantibodies

In children who developed multiple autoantibodies, IA-2A, as a second-appearing autoantibody, conferred a significantly greater risk of progression to type 1 diabetes (adjusted-HR=3.08 95%CI=2.04-4.65, *P*<0.0001; **Table 2, Figure 2)** as compared to IAA or GADA. There was no statistical evidence that the appearance of ZnT8A as a second autoantibody as compared to IAA or GADA modified risk of progression to type 1 diabetes (adjusted-HR=0.81, 95%CI=0.51-1.29, *P*=0.37). Other covariates significantly associated with progression in children who developed multiple autoantibodies were age at the appearance of the first autoantibody (adjusted-HR=0.98, 95%CI=0.97-0.99), *P*=0.001), time in months from the first to second autoantibody (adjusted-HR=0.97, 95%CI=0.96-0.99, *P*=0.003), and sex of the child (female versus male, adjusted-HR=1.52, 95%CI=1.09-2.13, *P*=0.014). Further assessment of specific HLA class I alleles and non-HLA SNPs (including interactions) previously shown to be associated with type 1 diabetes were not found significantly associated with progression in this population with increased HLA-DR-DQ genetic risk.

Time effect of first- to second-appearing autoantibody on risk of type 1 diabetes

Finally, we examined if the time duration between the first and second autoantibody influenced the risk of progression to type 1 diabetes after the development of the second autoantibody. The risk of progression from the second-appearing autoantibody to type 1 diabetes declined with increasing time duration in months between the first- and second-appearing autoantibody (for every additional month longer, adjusted-HR=0.97, 95%CI=0.96-0.99, *P*=0.006) (**Table 2**). Such that, there was a 2-fold increased risk of progression to type 1 diabetes in children who developed a second autoantibody within 1 year of their first-appearing autoantibody as compared to those who developed it after 1 year post initial seroconversion (adjusted-HR=2.24 95%CI=1.45-3.46, *P*=0.0003).

Discussion

In this large cohort of children at increased genetic risk for type 1 diabetes with either IAA or GADA as a first-appearing autoantibody, we found that the appearance of a second autoantibody, regardless of type, conferred at least a 5-fold increased risk of progression to type 1 diabetes as compared to children who remained single autoantibody positive. This increased risk of progression varied by type of second-appearing autoantibody with IA-2A conferring the highest risk as compared to GADA, IAA or ZnT8A and was altered by how rapid the second autoantibody appeared. Development within one year doubled the risk of progression to type 1 diabetes. Furthermore, a younger age at the initial seroconversion increased the risk of developing a second autoantibody and type of second-appearing autoantibody was independent of type of first-appearing autoantibody. Our results showed that having a family member with type 1 diabetes at time of screening (sibling, father or mother) was associated with the risk of a specific second autoantibody. And, carrying HLA-DR3/3 or the minor allele in rs1004446, a SNP in the *INS-VNTR* gene region, decreased the chance of IAA or GADA as a second-

appearing autoantibody. Knowing the type of second autoantibody and how quickly it develops would increase our ability to identify subjects who are more likely to have a rapid versus slow progression to type 1 diabetes.

The strengths of this study include the ability to assess risk of the appearance of another autoantibody and the temporal impact of this second autoantibody after the time of initial seroconversion. Previous studies have focused on assessing the risk of combinations of autoantibodies in cross-sectional screening designs when actual timing of initial seroconversion was unknown or in longitudinal studies with varied screening intervals or at/near diagnosis with type 1 diabetes in those with familial risk. The relatively short interval for autoantibody screening in this study captured the sequential time of autoantibody appearance in TEDDY children from both the general population (89%) and those with a family history.

The majority of TEDDY children (77%) developed first-appearing IAA or GADA very early in life (7), as also observed in the Finnish DIPP study (8). Consistent with other studies (6; 8; 9), both IA-2A and ZnT8A were less likely to be a first-appearing autoantibody. Overall, ZnT8A appeared around a median of 3-4 years of age in the BABYDIAB (11) and 4-5 years of age in TEDDY, a difference likely due to the predominance of first degree relatives in the former and a general population with increased genetic risk in the latter. The reason for this later appearance of ZnT8A is not clear; however, previous studies have reported that ZnT8A is frequently detected in those who progress to type 1 diabetes more slowly (21; 39). In TEDDY, GADA or IAA were more likely to present as a second-appearing autoantibody in children under 10 years of age. It cannot be excluded that the perceived delay in the appearance of a specific second autoantibody as it relates to the age when the first autoantibody was triggered could mark

a more environmentally driven, such as a prolonged enterovirus infection (40), as compared to a genetically driven disease process.

Unlike IAA, GADA and IA-2A, questions remain as to how much ZnT8A contributes to risk of Stage 3 type 1 diabetes, particularly in those who are young with increased genetic risk. In this study, ZnT8A conferred a 5-fold greater risk of progression to type 1 diabetes as compared to those autoantibody positive children who did not develop multiple autoantibodies. It is evident from our finding and other studies that ZnT8A can stratify risk of type 1 diabetes progression in familial risk (11; 14; 32), ethnically diverse (41), and young, high genetic risk populations with or without familial risk (TEDDY). However, a large study of ICA-positive relatives of type 1 diabetes probands did not find ZnT8A a contributing marker of type 1 diabetes in those <20 years at increased genetic risk (22). The likely reason for this inconsistency with our finding may be due to the preference of IAA, GADA or IA-2A over ZnT8A in those with familial and genetic risk, the use of cytoplasmic islet cell autoantibodies vs. IAA, GADA and IA-2A in risk determination, the high degree of concordance between GADA and ICA and the small number (n=69) of subjects with increased genetic risk less than 20 years of age (22).

Understanding the transition from single autoantibody positive to Stage 1 (multiple autoantibodies) has both research and clinical implications. It has been shown in multiple studies that the first-appearing autoantibody is age-dependent and likely associates with different environmental triggers (5; 7-9; 33). Assessment of previously published environmental risk factors (34-37) shown to associate with the induction of islet autoimmunity in TEDDY were not found to be involved in further development of a second autoantibody. We, therefore, suggest that an environmental factor(s), such as prolonged viral shedding (40), may be sought as the aetiological agent for the autoimmune reaction that is triggering the appearance of the first

autoantibody and thereby the commencement of the autoimmune pathogenesis. The present analysis suggests that the appearance of a second autoantibody is related to other yet unknown mechanisms often referred to as epitope spreading. The present data expand our understanding of the temporal pattern by which the second autoantibody appears. Although the mechanisms need to be clarified, at least it improves prediction of progression to type 1 diabetes, which advances our clinical practices for those at greatest risk and recruitment to secondary prevention clinical trials (42). Ultimately, this will result in better age-dependent autoantibody screening approaches, a reduction in symptomatic diagnoses and more strategic treatment interventions. Such specific autoantibody risk profiling will reduce heterogeneity and improve selection criteria to evaluate study objectives and interventions.

The main limitations are the select high HLA-DR-DQ genetic risk population, lack of extended follow-up past the age of 10 years and the screening protocol for ZnT8A, which only measured ZnT8A in those children who developed another autoantibody (GADA, IA-2A or IAA) further limiting our ability to determine the incidence of ZnT8A as a first appearing autoantibody. A potential weakness of the present study is the inclusion criteria of children in four different countries with increased genetic risk for diabetes (26). The HLA-DR-DQ genotypes included 4-7.5% of newborns. However, the expected number of children reaching type 1 diabetes by 18 years of age would represent less than 50% of the total number of children developing type 1 diabetes. Certain HLA-DQ haplotypes, such as DQ5.1 and DQ6.4 are missing from the TEDDY cohort. It cannot be excluded that the pattern of IA-2A and ZnT8A as a second-appearing autoantibody may differ from the present observations.

Taken together, in children less than 10 years of age with genetic risk for type 1 diabetes, the type of second-appearing autoantibody was associated with both genetic and familial risk, as

well as, varying risks of progression to type 1 diabetes. All four autoantibodies under study increased the risk of Stage 3 type 1 diabetes. Age continues to play a critical role in the initiation of autoimmunity, development of multiple autoantibodies and progression to type 1 diabetes. The importance of understanding what triggers epitope spreading and the type of autoantibody is critical in prevention of disease progression. Further analyses including the adolescent years (>10 years) are required to better understand the transition to Stage 1 (multiple autoantibodies) and how it affects risk of Stage 3 type 1 diabetes, especially, in a more diverse general population with low to medium genetic risk.

Figure Legends

Figure 1. Cumulative incidence in children with distinct single first-appearing autoantibody positivity (IAA or GADA) for progression to distinct single second-appearing autoantibody positivity. Panel A shows the cumulative incidence of the second-appearing autoantibody by months since first-appearing IAA. Panel B shows the cumulative incidence of the second-appearing autoantibody by months since first-appearing GADA. The blue line represents IAA, black line represents GADA, red line represents IA-2A and green line represents Znt8A.

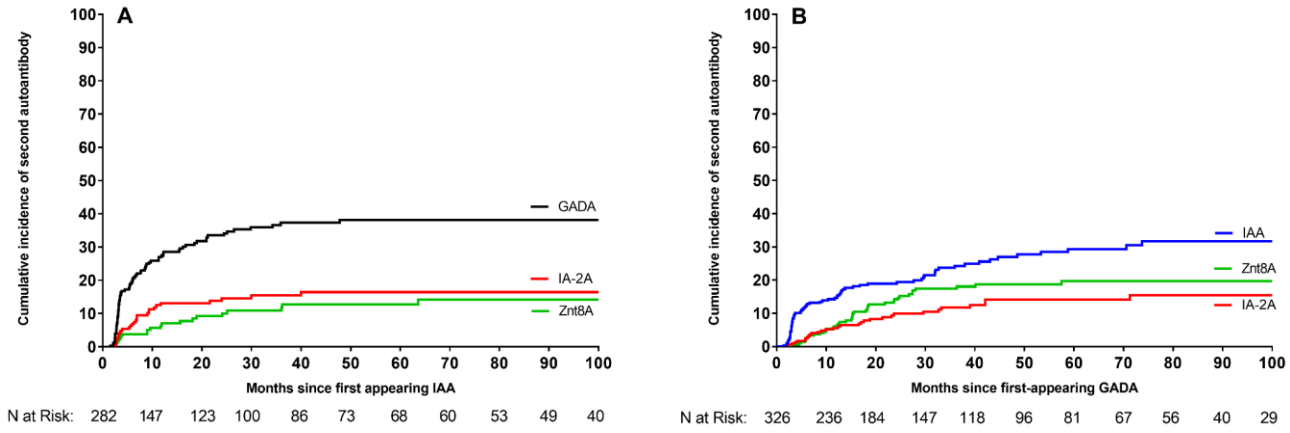
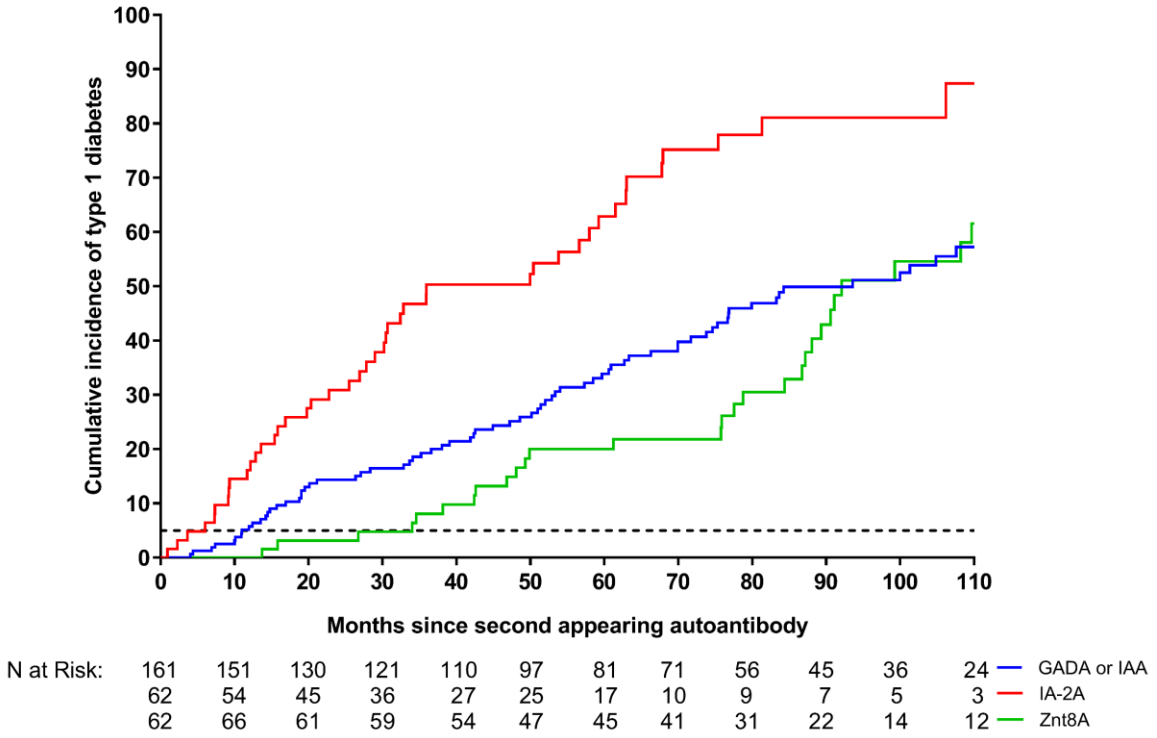


Figure 2. Cumulative incidence in children with a distinct single second-appearing autoantibody for progression to type 1 diabetes (blue line represents GADA or IAA, red line represents IA-2A, and green line represents ZnT8A). The black dotted horizontal line is the 5 year (60 month) cumulative risk of type 1 diabetes among the children who remained single autoantibody positive. The median (IQR) time between the first- and second-appearing autoantibody seroconversions was 5.3(3.0-13.3) months for GADA or IAA, 6.9(3.7-17.6) months for IA-2A, and 13.3(6.3-23.7) months for ZnT8A.



Author Contributions: All authors attest to meeting ICMJE uniform requirements for authorship by making substantial contributions to conception and design of this paper, acquisition, analysis and interpretation of the data, drafting or revising the article for intellectual content, and giving final approval of the published version. K.V. designed the study, proposed

the analysis, performed the analysis and contributed to the manuscript, interpreted the findings, and wrote the manuscript. Å.L., E.B., J.K. designed the study, interpreted the findings and reviewed/edited manuscript. J-X.S., J.T., A.Z., W.H., D.S., M.R. and B.A. designed the study and reviewed/edited manuscript. L.Y. A.W. reviewed/edited manuscript. K.V. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1. Multivariable cause-specific hazards ratios (CHR) in children with a single first-appearing IAA or GADA positivity (n=608) for progression to second autoantibody positivity (n=336ǂ).

	Second-appearing autoantibody		
	GADA or IAA	**IA-2A**	**ZnT8A**
	Cause-specific HR* (95% CI), *P*		
Female vs. Male	0.90(0.65-1.23), p=0.50	1.12(0.67-1.87), p=0.67	0.64(0.38-1.07), p=0.09
Family History of type 1 diabetes (reference= no family history)			
Sibling with type 1 diabetes	**2.22(1.12-4.40), p=0.023**	0.86(0.12-6.43), p=0.89	1.93(0.57-6.48), p=0.29
Father with type 1 diabetes	1.35(0.82-2.23), p=0.24	1.54(0.71-3.35), p=0.27	**2.15(1.04-4.46), p=0.039**
Mother with type 1 diabetes	0.80(0.31-2.10), p=0.65	**2.88(1.06-7.84), p=0.039**	2.15(0.66-7.08), p=0.21
HLA-DR-DQ genotype (reference=DR4/4)			
DR3/4	1.26(0.83-1.93), p=0.28	1.21(0.58-2.52), p=0.62	1.20(0.56-2.54), p=0.64
DR4/8	0.55(0.29-1.01), p=0.05	1.74(0.76-4.01), p=0.19	1.01(0.40-2.57), p=0.98
DR3/3	**0.32(0.16-0.67), p=0.003**	0.69(0.26-1.85), p=0.46	0.96(0.40-2.30), p=0.93
First Degree Relative (FDR) Specific**	1.01(0.35-2.89), p=0.98	0.38(0.04-3.30), p=0.38	NA, p=0.98
SNP rs1004446_A (*INS*)	**0.70(0.51-0.96), p=0.025**	0.94(0.56-1.57), p=0.81	1.03(0.62-1.72), p=0.90
First Appearing Ab (GADA vs. IAA)	0.94(0.67-1.33), p=0.73	1.23(0.69-2.20), p=0.48	1.62(0.91-2.86), p=0.09
Age at First Appearing Autoantibody	**0.99(0.98-0.99), p<0.0001**	**0.98(0.97-0.99), p=0.0002**	**0.99(0.98-99), p=0.003**

*Stratified Cox Regression with country of residence.

** FDR specific includes: DR4/1, DR4/9, DR4/13, DR3/9, DR4/4-DQB1*20X, DR4/4-DQB1*304.

ǂ 47 children developed two autoantibodies within the same 3-month interval as second-appearing autoantibodies and were censored at time of second-appearing autoantibody positivity. Data not presented due to difficulty in determining order of appearance.

Table 2. Multivariable proportional hazard ratios (HR) in children with a distinct second-appearing autoantibody (n=289) for progression to type 1 diabetes (n=149).

	HR (95% CI)	*P*
Type of second antibody		
IAA or GADA	1 [Reference]	
IA-2A	3.08 (2.04-4.65)	<0.0001
ZnT8A	0.81 (0.51-1.29)	0.37
Age at first-appearing autoantibody (months)	0.98 (0.97-0.99)	0.001
Time from first to second autoantibody (months)	0.97 (0.96-0.99)	0.003
Sex		
Female	1.52 (1.09-2.13)	0.014
Male	1 [Reference]	
Family History of T1D		
Mother with T1D	1.55 (0.65-3.67)	0.32
Father with T1D	1.01 (0.60-1.70)	0.97
Sibling with T1D	0.92 (0.46-1.76)	0.82
General population (no T1D history)	1 [Reference]	
HLA-DR-DQ genotype		
DR3/4	1.70 (0.99-2.93)	0.06
DR4/4	1 [Reference]	
DR4/8	1.32 (0.69-2.54)	0.41
DR3/3	0.97 (0.44-2.15)	0.94
FDR-specific*	1.28 (0.35-4.63)	0.71
Type of first antibody		
GADA only	0.97 (0.66-1.43)	0.89
IAA only	1 [Reference]	

*FDR specific HLA-DR-DQ genotypes are DR4/4b, DR4/1, DR4/9, DR4/13, and DR3/9.